RECOMBINANT NUCLEIC ACID USEFUL FOR INDUCING PROTECTIVE IMMUNE RESPONSE AGAINST ALLERGENS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/406,659, filed August 29, 2002, the content of which is herein incorporated by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to recombinant nucleic acid useful for inducing protective immune response against allergens and to vaccines comprising the nucleic acid.

BACKGROUND OF THE INVENTION

[0003] A dramatic increase in the prevalence of allergic diseases worldwide in recent years, particularly in developing countries such as the US, Western Europe, Australia, Japan and Singapore, has highlighted the need for new therapeutic and preventive medical reagents and strategies aimed at suppressing or redirecting the immune response induced upon exposure of an atopic individual to an allergen (1-8).

[0004] Briefly, activation of an immune response requires the activation of T cells, either cytotoxic T (killer) cells, or T helper cells. Cytotoxic T cells (commonly referred to as CD8+ cells) are responsible for cellular-based immunity. These cells are stimulated by the presentation of antigen epitopes in complex with MHC class I molecules at the surface of an antigen presenting cell. Antigen-activated cytotoxic T cells then induce cytosis of infected cells presenting the specific antigen epitope. Antigens that enter the MHC class I presentation pathway are usually derived from pathogens that multiply within the cytoplasm of a host cell, such as a virus.

[0005] T helper cells (commonly referred to as CD4+ cells) are involved in humoral immunity. T helper cells are activated by the presentation of antigen

a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene fragment encoding H-2^d-restricted Th epitope and the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Blo t 5 protein.

[0020] **Figure 4** shows induction of specific Th1 type humoral response in BALB/cJ mice via intradermal injection with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene fragment encoding H-2^d-restricted Th epitope and the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Blo t 5 protein.

[0021] **Figure 5** shows induction of specific Th1 type humoral responses in BALB/cJ mice via intramuscular injection with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene fragment encoding H-2^d-restricted Th epitope and the LAMP-1 transmembrane and cytoplasmic domain, followed by boosting with alum-absorbed Blo t 5 allergen protein and subsequent aerosol administration of Blo t 5 allergen protein.

[0022] **Figure 6** shows induction of long-term Blo t 5-specific immunity memory in BALB/cJ mice intramuscularly injected with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene fragment encoding H-2^d-restricted Th epitope and the LAMP-1 transmembrane and cytoplasmic domain, and then boosted with alum-absorbed Blo t 5 allergen protein after a prolonged interval.

[0023] **Figure 7** shows induction of long-term Blo t 5-specific immunity memory in BALB/cJ mice intramuscularly injected with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene fragment encoding H-2^d-restricted Th epitope and the LAMP-1 transmembrane and cytoplasmic domain, and then boosted with alum-absorbed Blo t 5 allergen protein. The DNA vaccine priming was given in three doses over an extended period of time before boosting was performed.

[0024] **Figure 8** shows induction of specific Th1 humoral immune responses in BALB/cJ mice by intramuscular injection with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Blo t 5* gene and with or without the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Blo t 5 protein.

[0025] **Figure 9** shows the induction of Der p 1-specific Th1 type immunity in BALB/cJ mice by intramuscular injection with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Der p 1* gene and the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Der p 1 protein.

[0026] **Figure 10** shows the suppression of *Der p* 1-specific Th2 cytokine production and the inhibition of airway hyperreactivity to *Der p* 1 in BALB/cJ mice by gene immunization. Immunization was done by intramuscular injection with a DNA vaccine encoding a chimeric protein comprising the LAMP-1 signal sequence, the *Der p 1* gene and the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Der p 1 protein.

[0027] **Figure 11** shows the production of Th1 specific antibodies raised against Der p 1 in BALB/cJ mice immunized intramuscularly with a DNA vaccine encoding a chimeric protein comprising the human tissue plasminogen activator signal sequence, the *Der p 1* gene and and the LAMP-1 transmembrane and cytoplasmic domain, and subsequent boosting with alum-absorbed Der p 1 protein.

[0028] **Figure 12** shows the production of Th1 specific antibodies raised against Der p 1 in BALB/cJ mice immunized orally with chitosan-DNA nanoparticles. The nanoparticles contained a DNA vaccine encoding a chimeric protein comprising the human tissue plasminogen activator signal sequence, the *Der p 1* gene and and the LAMP-1 transmembrane and cytoplasmic domain. Priming was followed by

signal peptide may be the N-terminal signal sequence from the gene for LAMP-1, human tissue plasminogen activator (see for example SEQ ID NO: 49), lysosomal membrane protein LIMP-II (see for example SEQ ID NOS:8, 10, 12, 28, 30, 32), ($CD4^+$ T Cells Induced by a DNA Vaccine: Immunological Consequences of Epitope-Specific Lysosomal Targeting. Fernando Rodriguez, Stephanie Harkins, Jeffrey M. Redwine, Jose M. De Pereda, And J. Lindsay Whitton. JOURNAL OF VIROLOGY, Vol. 75(21): 10421–10430.2001; The Residues $Leu(Ile)^{475}$-Ile(Leu, Val, $Ala)^{476}$, Contained in the Extended Carboxyl Cytoplasmic Tail, Are Critical for Targeting of the Resident Lysosomal Membrane Protein LIMP I1 to Lysosomes. Ignacio V. Sandoval Juan J. ArredondoS, Jose Alcalde, Alfonso Gonzalez Noriegall, Joel Vandekerckhove, Maria A. Jimenezll, and Manuel Rico. The Journal of Biochemistry, Vol. 269(9): 6622-6631,1994; Targeting of Lysosomal Integral Membrane Protein LIMP I1 THE TYROSINE-LACKING CARBOXYL CYTOPLASMIC TAIL OF LIMP I1 IS SUFFICIENT FOR DIRECT TARGETING TO LYSOSOMES. Miguel A. Vega, Fernando RodriguezSV, Bartolome Segui, Carmela Calesll, Jose Alcalde, and Ignacio V. Sandoval. THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 266(25): 16269-16272,1991; Cloning, Sequencing, and Expression of a cDNA Encoding Rat LIMP 11, a Novel 74-kDa Lysosomal Membrane Protein Related to the Surface Adhesion Protein CD36. Miguel A. Vega, Bartolome Segui-Real, Jose Alcalde Garcia, Carmela Cales, Fernando Rodriguez, Joel Vanderkerckhovev, and Ignacio V. Sandoval. THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 266(25): 16818-16824,1991), DEC-205 (see for example SEQ ID NOS:14, 16, 34, 36) (The Dendritic Cell Receptor for Endocytosis, DEC-205, Can Recycle and Enhance Antigen Presentation via Major Histocompatibility Complex Class II–positive Lysosomal Compartments Karsten Mahnke, Ming Guo, Sena Lee, Homero Sepulveda, Suzy L. Swain, Michel Nussenzweig, and Ralph M. Steinman. The Journal of Cell Biology, Vol. 151(3): 673–683,2000; Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral $CD8^+$ T Cell Tolerance. Laura Bonifaz, David Bonnyay, Karsten Mahnke, Miguel Rivera, Michel C. Nussenzweig, and Ralph M. Steinman. J. Exp. Med. Vol. 196(12):

MHC class I molecules, activate CD8+ T cells capable of conferring protection against subsequent allergic challenge (see US patent nos. 5,958.891 and 6,251,663, and Kwan et al). In contrast, there was no suggestion that enhancing MHC class II presentation or processing would be advantageous in inhibiting IgE production.

[0035] In the present invention, the inventors have made the surprising discovery that targeting an allergen to MHC class II processing and presentation pathway in the vaccination group can induce a strong Th1 immune response, mediated by IgG_{2a}, while inhibiting Th2 immune response as mediated by IgE when compared to a control group. The inventors have further found that a signal sequence that mediates the translocation of allergen once expressed in the cell to the endoplasmic reticulum is sufficient to induce a Th 1 immune response. Without being limited to any particular theory, it is believed that once in the endoplasmic reticulum, at least some of the allergen is routed to MHC class II processing and presentation.

[0036] Preferably, the recombinant DNA further comprises an operably linked gene encoding a second signal peptide wherein the second signal peptide targets the allergen to an endosome or lysosome. This is believed to further enhance presentation of the allergen in the MHC class II pathway. The gene encoding the second signal peptide may be any sequence that encodes an amino acid sequence that interacts with the cell machinery to target the allergen to which it is attached to a lysosome or an endosome. For example and without limitation, the second signal peptide may be the C-terminal lysosomal/endosomal targeting sequence from the gene for LAMP-1, human tissue plasminogen activator, LIMP-II (see for example SEQ ID NOS:9, 11, 13, 29, 31, 33), DEC-205 (see for example SEQ ID NOS:15, 17, 35, 37), P-selectin (see for example SEQ ID NOS:19, 39), human tyrosinase (see for example SEQ ID NOS:21, 41), the glucose transporter GLUT4 (see for example SEQ ID NOS:23, 43), endotubin (see for example SEQ ID NOS:25, 45) or Nef protein, or a functional equivalent meaning any variation in the sequence that does not affect its function of targeting to an endosome or lysosome, for example allelic variants, conservative amino acid substitutions and substantially homologous sequences as described in

[0042] Where an amino acid is represented by more than one codon in the genetic code, a given organism may exhibit a particular preference or more common usage of one codon over another. For example, the codons AGG, AGA and CGT all encode arginine. AGG and AGA are used frequently in human coding sequences, while codon CGT is rarely used. Thus, silent mutations within a coding region of DNA made to select a codon preferred for a particular organism, but which result in expression of the same amino acid sequence of an allergen, are included within the scope of the invention and the term "humanized" is used to refer to changes in the gene sequence to select for codons preferred or commonly found in human coding sequences.

[0043] The term gene is used in accordance with its usual meaning to mean an operably linked group of nucleic acid sequences. The term recombinant means that something has been recombined such that reference to a recombinant nucleic acid refers to a molecule that is comprised of nucleic acid sequences that are joined together or produced by means of molecular biological techniques. A first nucleic acid sequence is operably linked to a second nucleic acid sequence when the sequences are placed in a functional relationship. For example, a coding sequence is operably linked to a promoter if the promoter activates the transcription of the coding sequence. Similarly, the gene encoding the first signal peptide is operably linked to the gene coding the allergen if upon expression of the recombinant DNA, the signal peptide mediates the translocation of the allergen to the endoplasmic reticulum. Similarly, the gene coding the second signal peptide is operably linked if upon expression of the recombinant DNA the second signal peptide targets the allergen to an endosome or lysosome.

[0044] In one embodiment, the gene encoding the first signal peptide is operably linked upstream to the gene encoding the allergen and the gene encoding the second signal peptide is operably linked downstream from the gene encoding the allergen. In specific embodiments, the recombinant DNA comprises one or more sequences of SEQ ID NOS. 3 to 7 and 28 to 48.

are administered in the first phase over a period of about a year.

[0079] The allergen may be administered in the second phase in one or more doses in combination with an adjuvant. Preferably, the adjuvant is chosen so as to elicit allergen-specific Th type 1 immune response. Such a response may be measured by the production of Th1 specific immunoglobulins and cytokines. In one embodiment, the allergen is administered in combination with alum.

[0080] The amount of allergen and adjuvant to be administered can be determined by routine experimentation by a skilled person. In one embodiment, about 100 ng and 1 mg of allergen is administered, preferably about 1 μg to 100 μg. In a further embodiment, the allergen is administered in combination with about 1 mg to 10 mg of adjuvant, preferably about 2 mg to 5 mg of adjuvant. The allergen or allergen plus adjuvant may be administered by methods commonly known in the art. For example, administration may be oral, sub-lingual, intraperitoneal, nasal, intratracheal, intramuscular, sub-cutaneous, intradermal, etc.

[0081] The allergen in the second phase may administered in one or more doses. The second phase may occur immediately following the first phase, or there may be an interval of time between the last administration of nucleic acid in the first phase and the initiation of administration of allergen in the second phase. If multiple doses of allergen are given, the doses may be administered over a given time span by different administration routes. For example, two or more doses may be administered in a period of two days up to about 10 weeks. The timing of the administration of the doses may be evenly spaced over the time span, or the doses may be given at irregular intervals over the time span.

[0082] In one embodiment, the method comprises administration of at least one dose of the allergen by aerosol, preferably, the last dose is given by aerosol.

[0083] Thus, in one embodiment of the immunization regimen, Th1 type allergen-

[0095] The control vector pCI-LAMPss-T/C was constructed by insertion of the synthetic oligonucleodide composing the LAMP-1 leader sequence and the LAMP-1 sequence encoding the transmembrane and cytoplasmic tail into the *Xho I* (the corresponding site in the insert is bolded at the 5' end of sequence below) and *Not I* (the corresponding site in the insert is bolded at the 3' end of sequence below) of pCI vector. A unique *Nhe I* site and a unique *Nde I* site were designed at the 3'end of sequence encoding the LAMP-1 leader sequence and at the 5'end of encoding sequence for LAMP-1 transmembrane and cytoplasmic tail, respectively (both underlined). The encoding sequence of the pCI-LAMPss-T/C with the cloning sites into which an allergen gene is inserted is shown below [SEQ ID NO:1]. The translated protein sequences for the mouse LAMP-1 leader sequence and the mouse LAMP-1 transmembrane and cytoplasmic domain are also shown [SEQ ID NO:25, SEQ ID NO:26]:

M A A P G A R R P L L L L L L A G L A H G

5' **ctcgag**ccaccatggccgccccggcgcccggaggcccctgctcctgctgctggcaggccttgcacatggc

A S M L I P I A V G G A L A G L V L

gctagcgaattcccggggatccatatgttgatccccattgctgtgggcggtgccctggcagggctggtcct

I V L I A Y L I G R K R S H A G Y E T I

atcgtcctcatcgcctacctcattggcaggaagaggagtcacgccggctatcagaccatctag**cggccgc** 3'

[0096] Plasmid pCI-LAMPss-Bt5_{50-67}-T/C was constructed using synthetic oligonucleotide composing the Blo t 5 gene fragment that encodes for the H-2^d-restricted Th epitope. The oligonucleotide was inserted into the *Nhe I* site at the 3' end of the LAMP-1 leader sequence and the *Nde I* site at the 5' end of the LAMP-1 sequence encoding the transmembrane and cytoplasmic tail. The encoding sequence is [SEQ ID NO:2]:

Mouse LAMP-1 signal sequence
M A A P G A R R P L L L L L L A G L A H G A S
5' atggccgcccccggcgcccggaggcccctgctcctgctgctggcaggccttgcacatggcgctagc 3'

Blo t 5 H-2^d-restricted T cell epitope
A E L Q E K I I R E L D V V C A M N
5' gcagaattgcaagagaaaatcattcgagaacttgatgttgtttgcgccatgaat 3'

Mouse LAMP-1 transmembrane & cytoplasmic domain
M L I P I A V G G A L A G L V L I V L I A Y L
5' atgttgatccccattgctgtgggcggtgccctggcagggctggtcctcatcgtcctcattgcctacctc

Mouse LAMP-1 transmembrane & cytoplasmic domain
I G R K R S H A G Y E T I AMB
attggcaggaagaggagtcacgccggctatcagaccatctag 3'

[0097] Plasmid pCI-LAMPss-Bt5-T/C was generated by insertion of PCR amplified *Blo t 5* cDNA encoding the mature protein into the *Nhe I* site at the 3' end of the LAMP-1 leader sequence and the *Nde I* site at the 5' end of the LAMP-1 sequence encoding the transmembrane and cytoplasmic tail. The Blo t 5-LAMP encoding sequence is [SEQ ID NO:3]:

Mouse LAMP-1 signal sequence
M A A P G A R R P L L L L L L A G L A H G A S
5' Atggccgcccccggcgcccggaggcccctgctcctgctgctggcaggccttgcacatggcgctagc 3'

Blo t 5 encoding sequence
Q E H K P K K D D F R N E F D H L L I E Q A N H
5' caagagcacaagccaaagaaggatgatttccgaaacgaattcgatcacttgttgatcgaacaggcaaaccat

A I E K G E H Q L L Y L Q H Q L D E L N E N K S
gctatcgaaaagggagaacatcaattgctttacttgcaacaccaactcgacgaattgaatgaaaacaagagc

K E L Q E K I I R E L D V V C A M I E G A Q G A
aaggaattgcaagagaaaatcattcgagaacttgatgttgtttgcgccatgatcgaaggagcccaaggagct

L E R E L K R T D L N I L E R F N Y E E A Q T L
ttggaacgtgaattgaagcgaactgatcttaacattttggaacgattcaactacgaagaggctcaaactctc

S K I L L K D L K E T E Q K V K D I Q T Q N

agcaagatcttgcttaaggatttgaaggaaaccgaacaaaaagtgaaggatattcaaacccaaaat 3'

Mouse LAMP-1 transmembrane & cytoplasmic domain
M L I P I A V G G A L A G L V L I V L I A Y L I
5' atgttgatccccattgctgtgggcggtgccctggcagggctggtcctcatcgtcctcatcgcctacctcatt

G R K R S H A G Y E T I
ggcaggaagaggagtcacgccggctatcagaccatctag 3'

[0098] Plasmid pCI-LAMPss-Bt5 was derived from pCI-LAMPss-Bt5-T/C by replacement of the *Eco RI /Not I* fragment encoding for a portion of Blo t 5 and the LAMP-1 transmembrane and cytoplasmic domain with the *Eco RI/Not I* fragment from pCI-Bt5. The encoding sequence is [SEQ ID NO: 4]:

Mouse LAMP-1 signal sequence
M A A P G A R R P L L L L L L A G L A H G A S
5' Atggccgcccccggcgcccggaggcccctgctcctgctgctggcaggccttgcacatggcgctagc 3'

Blo t 5 encoding sequence
Q E H K P K K D D F R N E F D H L L I E Q A N H
5' caagagcacaagccaaagaaggatgatttccgaaacgaattcgatcacttgttgatcgaacaggcaaaccat

A I E K G E H Q L L Y L Q H Q L D E L N E N K S
gctatcgaaaagggagaacatcaattgctttacttgcaacaccaactcgacgaattgaatgaaaacaagagc

K E L Q E K I I R E L D V V C A M I E G A Q G A
aaggaattgcaagagaaaatcattcgagaacttgatgttgtttgcgccatgatcgaaggagcccaaggagct

L E R E L K R T D L N I L E R F N Y E E A Q T L
ttggaacgtgaattgaagcgaactgatcttaacattttggaacgattcaactacgaagaggctcaaactctc

S K I L L K D L K E T E Q K V K D I Q T Q N
agcaagatcttgcttaaggatttgaaggaaaccgaacaaaaagtgaaggatattcaaacccaaaattaa 3'

[0099] Plasmid pCI-LAMPss-Derp1-T/C was generated by insertion of PCR-amplified Der p1 fragment encoding for the mature *Der p 1* protein (ref. 20. The gene bank access number is U11695) into the *Nhe I* site at the 3' end of the LAMP-1 leader sequence and the *Nde I* site at the 5' end of the LAMP-1 sequence encoding the

tail from LAMP-1 into the BamH I and Xba I sites of pVax (Invitrogen) which is a plasmid vector approved by the FDA for human use. The encoding sequence is [SEQ ID NO: 6]:

human tissue plasminogen activator leader sequence
5' atg gat gca atg aag aga ggg ctc tgc tgt gtg ctg ctg ctg tgt gga gca gtc ttc gtt tcg ccc agc cag gtt ggt gtg cag gac ccc tgt gtc ccg ccc ctc 3'

humanized Der p 1 sequence
5' acc aac gcc tgc agc atc aac ggc aat gcc ccc gct gag att gat ctg cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg tct tgt tgg gcc ttc tca ggc gtg gcc gcg acc gag tct gca tac ctc gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac tgc gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac gta gct agg gag cag tcc tgc cgc cgt cct aac gcc cag cgc ttc ggc att tcc aac tat tgc cag atc tac ccc cct aat gtg aac aag atc agg gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag cgc gac aac gga tac cag cca aac tat cac gcg gtc aac atc gtg ggt tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg cgg aac agt tgg gac acc aac tgg ggc gac aac ggc tac ggc tac ttt gcc gcc aac atc gac ctg atg atg atc gaa gag tac ccg tac gtg gtg atc ctg 3'

LAMP-1 transmembrane and cytoplasmic domain
5' ttg atc ccc att gct gtg ggc ggt gcc ctg gca ggg ctg gtc ctc atc gtc ctc att gcc tac ctc att ggc agg aag agg agt cac gcc ggc tat cag acc atc tag 3'

[00101] Production of recombinant Blo t 5 allergen and purification of native Der p 1: Two different expression systems, the *E.coli* based GST Gene Fusion System and the yeast based *Pichia* Expresssion System were employed to express the recombinant *Blot 5* allergen. For the *E.coli* based expression system, the entire encoding sequence for mature *Blo t 5* was subcloned into the vector pGEX-4T (Amersham Phamacia Biotech). In order to obtain recombinant *Blo t 5* with post-translation modification properties of the native *Blo t 5*, the coding sequence for the mature *Blo t 5* was subcloned into the pPICZα vector using the EasySelect™ Pichia Expression Kit (Invitrogen™ life technologies). Protein expression and purification were achieved according to the manual provided by the manufacturers. Native Der p 1 was purified from spent mite media using mAb 4C1 by affinity chromatography.